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# Live-cell imaging of membrane proteins by a coiled-coil labeling method—Principles and applications

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*Abbreviations:* AFM, atomic force microscopy;  $\beta_2$ AR,  $\beta_2$ -adrenergic receptor; BRET, bioluminescent resonance energy transfer; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FRET, fluorescence resonance energy transfer; GpA, glycophorin A; GPCR, G-protein-coupled receptor; M2, M2 protein of influenza A virus; SDS, sodium dodecyl sulfate

## Abstract

In situ investigations in living cell membranes are important to elucidate the dynamic behaviors of membrane proteins in complex biomembrane environments. Protein-specific labeling is a key technique for the detection of a target protein by fluorescence imaging. The use of post-translational labeling methods using a genetically encodable tag and synthetic probes targeting the tag offer a smaller label size, labeling with synthetic fluorophores, and precise control of the labeling ratio in multicolor labeling compared with conventional genetic fusions with fluorescent proteins. This review focuses on tag–probe labeling studies for live-cell analysis of membrane proteins based on heterodimeric peptide pairs that form coiled-coil structures. The robust and simple peptide–peptide interaction enables not only labeling of membrane proteins by noncovalent interactions, but also covalent crosslinking and acyl transfer reactions guided by coiled-coil assembly. A number of studies have demonstrated that membrane protein behaviors in live cells, such as internalization of receptors and the oligomeric states of various membrane proteins (G-protein-coupled receptors, epidermal growth factor receptors, influenza A M2 channel, and glycopholin A), can be precisely analyzed using coiled-coil labeling, indicating the potential of this labeling method in membrane protein research.

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## 1. Introduction

Vital functions of cell membranes, such as signal transduction, material transport, energy conversion, and intercellular communication are mediated by integrated membrane proteins. The lipid bilayer structure of biomembranes provides a distinct solvent environment for integrated proteins, such as gradient changes in the chemical functional groups with different hydrophobicities (e.g. hydroxyl, choline, phosphate, carbonyl, and methylene groups), along with the direction of bilayer normal [1]. Furthermore, cell membranes contain several hundred or more different lipid species, varying in physicochemical properties such as electric charge and fluidity. The distribution of the lipids is heterogeneous among organelles [2,3]. Such lipid heterogeneity and organelle-specific features of protein transmembrane domains (e.g. hydrophobic lengths and amino acid compositions) imply regulation of protein activity by intracellular location [4]. Consistent with this, the lipid compositions of reconstituted proteoliposomes often dramatically alter the activity of incorporated membrane proteins [5]. Studies using model transmembrane helices have also revealed that the thermodynamic and dynamic properties of helix–helix interaction, the principal interaction that determines stability of membrane proteins, strongly depend on lipid composition [6-8].

Therefore, experimental approaches for the in situ investigation of membrane proteins in living cell membranes are essential to understand the behavior of the proteins related to their functions, in addition to reconstituted and other experimental systems containing isolated proteins.

Selective labeling and imaging methods are key to adequately observe the behaviors of target membrane proteins in live-cells. Fluorescence imaging is the most widely used technique for this purpose due to its potential high sensitivity (down to the single-molecule level) and spatio-temporal resolution ( $< 100$  nm in super-resolution microscopy and  $\sim 1$  msec with a high speed camera). Genetic fusion of fluorescent proteins to a target protein (Figure 1A) has been routinely used for protein-specific labeling and image analysis in living cells [9]. However, the large size of fluorescent proteins ( $\sim 27$  kDa) may disrupt the normal trafficking and function of target proteins [10,11]. In addition, precise control of the labeling ratio in multicolor labeling for fluorescence resonance energy transfer (FRET) measurements is not easy. To overcome these shortcomings, post-translational labeling methods using a genetically encodable tag and synthetic probes targeting the tag have been developed to specifically label proteins in living cells. The fluorescent moieties of the probes can have better photophysical properties, such as long-term photostability and greater brightness than fluorescent proteins. Figure 1B–E shows the pros and cons of tag–probe labeling methods so far reported based on protein–ligand interaction,

peptide–peptide interaction, peptide–metal–ligand interaction, and enzymatic reactions. In general, there is a trade-off between the label size and labeling specificity. Therefore, it is important to select labeling methods depending on the type of the experiment with sufficient specificity and minimal size. In practice, the availability of synthetic probes can also affect the feasibility of the labeling method. For example, peptide probes labeled with a fluorophore are generally available from companies offering custom peptide synthesis, while enzymes and substrates may not be easily available.

Another approach to obtain labeled proteins in live cells is the use of bio-orthogonal reactions with site-specific incorporation of bioorthogonal tags via genetic code expansion [12]. Label size of this method is extremely small. The synthetic probes are not necessarily easily available at present, however, the reaction rate can be fast (e.g. within 2 min) [13].

In this review, we focus on the labeling principle based on peptide–peptide assemblies that have coiled-coil structures [14,15]. The coiled-coil tag–probe labeling method has a good balance of small size (5–6 kDa, and minimally ~3 kDa in combination with acyl transfer labeling) and easy labeling procedure (simple, quick, and selective). After reviewing reported coiled-coil peptide sequences and labeling strategies, applications that demonstrate robust analysis of membrane protein behaviors will be described, such as internalization and self-association.

Details of sample preparation procedures for coiled-coil labeling can be found in our previous review [16]. Labeling chemistries used in the tag–probe labeling, including those related to the coiled-coil assembly, have recently been reviewed by Beck-Sickinger et al. [17]. Related reviews have also been published on selective labeling techniques available under live-cell conditions [18-21].

## 2. Principles of coiled-coil labeling

The coiled-coil is a protein structural motif consisting of two or more  $\alpha$ -helices that are wrapped around each other in a superhelical fashion [14,15]. The amino acid sequences contain a characteristic heptad repeat pattern  $(abcdefg)_n$ , where  $n$  is the number of repeats. Inspired by the coiled-coils found in natural proteins, *de novo* designed coiled-coils have also been extensively studied to understand the relationships among the sequence, structure, and stability [14]. Coiled-coil is stabilized by interstrand interactions such as hydrophobic interactions among interfacial amino acids at positions  $a$  and  $d$ , and salt bridges among amino acids at positions  $e$  and  $g$ . The amino acid sequence affects degree of oligomerization (dimer, trimer, or tetramer), oligomerization selectivity (homo- *versus* hetero- oligomerization), and helix orientation (parallel *versus* antiparallel).



## 2.1. Noncovalent labeling based on a coiled-coil assembly

In 2008, we reported coiled-coil tag–probe labeling, which is based on parallel heterodimeric coiled-coil formation between negatively-charged E<sub>n</sub> peptides (EIAALEK)<sub>n</sub> and positively-charged K<sub>n</sub> peptides (KIAALKE)<sub>n</sub> (n = 3 or 4) [22], originally designed by Litowski and Hodges [23]. In addition to electrostatic attractions, leucine zipper-type hydrophobic interactions at the interface drive tight heterodimer formation (Figure 2A). We found the E3 peptide to be suitable as the N-terminal extracellular tag of membrane proteins, and it was specifically labeled with a K3 or K4 peptide probe conjugated with fluorophores while retaining protein functions (Figure 2B) [22]. The labeling can be completed within 1 min, which is much faster than other tag–probe methods. Labeling of the E3 tag with the K3 probe ( $K_d \sim 60$  nM) is reversible and the probe can be washed out, whereas labeling with K4 is stronger ( $K_d \sim 6$  nM) and therefore suitable for long-term observation. Because the charged peptides are membrane-impermeable, the label is surface-specific (Figure 2B). Other reported coiled-coil probes also have surface specificity, although intracellular labeling is also possible by fusion with cell-penetrating polyarginine peptide [24]. Various fluorophores (e.g. fluorescein, TAMRA, ATTO 488, ATTO 565, Alexa 568, and

Alexa 647) are available for labeling, and are advantageous in multicolor labeling.

Xia et al. reported that CCE3 (E VAALEKE VAALEKE VAALEK)/CCK3 (K VAALKEK VAALKEK VAALKE) and their mutants were also useful as coiled-coil tag/probe for labeling membrane proteins [25]. In addition to a heterodimeric coiled coil (2-mer), a tag–probe pair based on a heterotrimeric coiled-coil (3-mer) of a GCN4 mutant has also been reported (tag: Ac-WG ALKKELE AAKKELE ALKKELA GGCGG ALEKELE ALEKEAE ALEKELA-NH<sub>2</sub>; probe Ac-GG ALKKKLE ALKKK-Dap(NBD)-E ALKKKLA-NH<sub>2</sub>) ( $K_d \sim 18$  nM) [26]. The tag sequence involves two coils connected with a GGCGG loop sequence. By virtue of the environment-sensitive fluorescence property of the fluorophore NBD positioned at the center of the probe sequence, enhancement of fluorescence intensity was observed following binding to the tag.

The above coiled-coil pairs for tag–probe labeling contain fairly regular repeat sequences with opposite net charges (negative tag/positive probe). On the other hand, Keating et al. reported computational design of coiled-coil interaction pairs (SINZIP peptides) that have irregular sequences and high selectivity [27-29]. Coil Y and Coil Z sequences, based on SINZIP-5 (NTVKELKNYIQELEERNAELKNLKEHLKFAKAELEFELAAHKFE) and SINZIP-6 (QKVAQLKNRVAYKLKENAKLENIVARLENDNANLEKDIANLEKDIANLERDVAR) (Figure 2C) were used as a tag–probe pair ( $K_d < 15$  nM) for labeling of live-cell membrane proteins [30].

Both sequences are available for the tag and probe due to the overall charge balance (net negative), although they are much larger (44 and 54 residues for SINZIP-5 and SINZIP-6, respectively) compared with E<sub>n</sub>/K<sub>n</sub> pairs (21 or 28 residues). Recently, a tag–probe pair with higher affinity (CoilE/CoilR,  $K_d = 13$  pM) has also been used for live-cell labeling of membrane proteins with nanoparticles for electron microscopy [31].

## 2.2. Covalent labeling guided by a coiled-coil assembly

Although in coiled-coil labeling efficient labeling is possible by a noncovalent interaction due to the small dissociation constants ( $K_d < 100$  nM) even without washout of free probes (Figure 3A), the coiled-coil assembly can be used as a guide for covalent labeling (Figures 3BC). Covalent labeling is useful for subsequent biochemical analysis such as electrophoresis after solubilization of the cells, in which noncovalent labeling may be lost. Covalent labeling can also be useful for single-molecule imaging of membrane proteins, that requires a high signal/background ratio under low expression levels of target proteins.

The accelerated crosslinking/acyl transfer reactions by the proximity effect, such as by the receptor–ligand interaction, have proven to be useful for covalent labeling under a

heterogeneous cell environment [32,33]. A number of studies have revealed that the coiled-coil assembly is also useful for proximity labeling of membrane proteins by cysteine-involved reactions ( $t_{1/2}$  of 1–15 min) [25,34-36]. Furthermore, the conventional sulfosuccinimidyl ester reaction is also useful for the selective labeling of the Lys of target tags on cells ( $t_{1/2}$  ~10 min ) [37]. In contrast to cysteine labeling, amine labeling does not require the reduction of cell-surface disulfides, thus simplifying the labeling procedure. Compared with crosslinking between the tag and probe (Figure 3B), acyl transfer reactions following coiled-coil assembly allow covalent labeling of the tag with minimal size (Figure 3C). Seitz and Beck-Sickinger et al. reported transfer of fluorophores attached at the N-terminus of K3 to Cys of E3, resulting in a small label (22 amino acids + fluorophore, ~3 kDa) [35,36,38]. The reaction proceeds in various neutral buffers and with high reactivity (85% yield after 5 min reaction time for the improved version) [36].

### 3. Analysis of behaviors of membrane proteins using coiled-coil labeling

#### 3.1. Subcellular localization

Surface-specific and quick labeling properties of coiled-coil labeling are useful for pulse

labeling of target proteins on the cell surface at the time of labeling. After the labeling, it is easy to track their internalization into endosomes by fluorescence imaging (Figure 4A). This is in contrast to imaging of fluorescent protein-fused proteins, in which both the cell surface and intracellular proteins exhibit fluorescence signals (Figure 2B). Membrane receptors are often desensitized and internalized after ligand stimulation. Several reports have observed internalization of membrane proteins labeled with the coiled-coil method, including  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR) [39,40], CXCR4 [41,42], neuropeptide  $Y_2$  receptor [36,38], and epidermal growth factor receptor (EGFR) [43]. Of note, the labeling did not deteriorate receptor signaling functions in these receptors, confirming minimal perturbation of the target proteins. A two-color pulse-chase labeling approach allowed the tracking of  $Y_2$ Rs in the same cell at different time points [38]. The ability to visualize the internalization pathway of two separately labeled and separately stimulated subsets of  $Y_2$ R in a time-resolved manner revealed rapid trafficking. Fusion of the two subsets was already observed 10 min after stimulation in the early endosomal compartment without subsequent separation of the fused receptor populations. The results demonstrate that the cells do not discriminate between receptors that were stimulated and internalized at different time points.

Two-color labeling of  $\beta_2$ ARs with pH-dependent and pH-independent fluorophores is useful for detecting acidification in endosomes following internalization. The degree of

translocation and acidification were evaluated with a cell image analyzer for ligand screening of  $\beta_2$ AR (Figure 4) [40]. In addition to internalization, surface-specific labeling is also used to detect surface expression of membrane proteins. Labeling examples have been reported for MHC-1 [44] and Hsp70 proteins [45].

Other than fluorophore labeling, E3/K3 coiled-coil interactions have been used for atomic force microscopy (AFM) detection of cell surface target proteins [46]. In this study, K3 peptides were linked to an AFM cantilever to detect E3-tagged target proteins (Hsp70). The cluster size distribution patterns were similar when Hsp 70 proteins were recognized by an anti-Hsp70 antibody, indicating the usefulness of E3/K3 systems in AFM imaging.

### 3.2. Oligomerization

Protein–protein interactions in membrane environments, and consequent formation of oligomers, are often crucial for the function of integral membrane proteins. For example, signaling of EGFR is triggered by homo/heterooligomerization between EGFRs or an EGFR and other ErbB family receptors, resulting in phosphorylation of intracellular tyrosine residues. Although it is clear that self-association is necessary for activation of EGFRs, their actual behavior

on cell membranes upon ligand binding is more complex than a simple transition from unliganded inactive monomers to liganded active dimers [47].

A more controversial topic is oligomerization of G-protein-coupled receptors (GPCRs), because the minimal functional unit of class-A GPCRs is a monomer (protomer) [48] and formation of homo/hetero oligomers may modulate their functions such as the ligand affinity and the type of coupled G-proteins.

The major obstacle to the study of the oligomerization–function relationship of membrane proteins is the lack of reliable measurement and analysis methods in live cell conditions. Detection of oligomerization by resonance energy transfer is direct evidence of close contact between receptors within ca. 5 nm. Although bioluminescent resonance energy transfer (BRET) is widely used to detect oligomers of membrane proteins, quantitative analysis is not easy, as exemplified in controversial results for self-association of  $\beta_2$ AR reported from different research groups [49,50], and questions regarding the significance of interactions between class-A GPCR protomers [51]. Use of a more precise method for analysis of receptor oligomerization is necessary to clarify this issue.

Important factors for quantitative analysis of FRET/BRET is the control of the donor/acceptor labeling ratio. Also, surface-selective detection may be essential to avoid signals

from intracellular aggregates. In 2006, Vogel and coworkers reported the self-association state of a neurokinin-1 receptor, a class-A GPCR, labeled with a tag–probe method (Acyl carrier protein tag, ~9 kDa) to precisely control the labeling ratio in FRET analysis [52]. The receptors were monomeric at the physiological expression level, but were suggested to be concentrated in membrane microdomains. Following this pioneering work, we have demonstrated that FRET analysis using coiled-coil labeling and confocal spectral imaging is also useful for stoichiometric analysis of the oligomeric state of membrane proteins with a smaller label size (Figure 5) [37,43,53-55]. Particularly, stoichiometric analysis is possible by analyzing FRET efficiencies at different labeling ratios (Figure 6). We determined the oligomeric states of class-A GPCRs ( $\beta_2$ AR, chemokine-CXCR4, dopamine-D2, and prostaglandin-EP1) by this method and found that these receptors did not form constitutive homooligomers, although some receptors formed clusters after ligand stimulation [53,55]. Overall, homooligomerization is not necessary for the function of these class-A GPCRs.

We also investigated the relationship between the oligomeric state and autophosphorylation of EGFR by this method, and observed epidermal growth factor (EGF)-dependent dimerization of EGFRs with only a minor fraction of predimers (~10%) [43]. In the process of activation, the presence of an inactive dimer that binds a single EGF molecule was



suggested, deduced from the difference in EGF concentrations that evoke half-maximal dimerization (~1 nM) and half-maximal autophosphorylation (~8 nM).

The M2 protein of the influenza A virus (M2) has been proposed to form an acid-activated, proton-selective ion channel that exhibits multiple functions upon viral infection. Although X-ray crystallography and NMR studies using transmembrane fragment peptides have suggested that M2 stably forms a tetrameric channel, the oligomeric state of the full-length protein in the cell was unknown. Our FRET analysis and channel activity measurements revealed that M2 formed proton-conducting dimers at neutral pH and that these dimers were converted to tetramers at acidic pH [54]. The antiviral drug amantadine hydrochloride inhibited both tetramerization and channel activity. The removal of cholesterol resulted in a significant decrease in the activity of the dimer. These results indicate that the minimal functional unit of M2 is a dimer.

Glycophorin A (GpA) is a well-studied single-pass transmembrane protein with dimerization properties in detergent micelles, driven by self-association of the transmembrane domains. By using a covalent labeling method following coiled-coil assembly, oligomeric states of GpA were compared in mammalian cells and sodium dodecyl sulfate (SDS) micelles [37]. In the cell membranes, no significant self-association of GpA was detected, whereas SDS-PAGE suggested partial dimerization of the proteins. Membrane cholesterol was found to be an

important factor that suppressed the dimerization of the proteins. Interestingly, we have shown that a model transmembrane helix having a self-association motif of GpA (GXXXG) also exhibited cholesterol-dependent suppression of dimerization, indicating an interplay between amino acid sequence and cholesterol regulates protein–protein interactions in the transmembrane region.

The coiled-coil method can also be used to control the oligomeric state of membrane proteins. Nakase, Futaki and colleagues developed an artificial dimerization system for E3-tagged EGF receptors using a bivalent K4 probe or K4-coated endosomes [56,57]. This approach enables studying receptor activation and signal transduction without native ligands, and is useful for artificial regulation of cellular signaling.

In summary, the above examples demonstrated that FRET analysis using coiled-coil labeling and spectral imaging is a powerful tool to elucidate precise oligomeric states of membrane proteins in a live cell environment. They also clarified the importance of membrane cholesterol on the oligomeric state of M2 and GpA proteins in biomembranes.

#### 4. Conclusions

Coiled-coil labeling is a simple, quick, and selective labeling method with moderate size for fluorescence imaging of live-cell membrane proteins. A robust coiled-coil assembly allows not

only noncovalent labeling, but also subsequent crosslinking and acyl transfer reactions under live cell conditions, extending the versatility of the labeling method. This labeling is useful for precise and quantitative analysis of membrane protein behaviors such as receptor internalization and oligomerization. Thus, the coiled-coil labeling method provides a versatile tool for studying live-cell membrane proteins.

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## Figure Legends

Figure 1 Schemes of genetic fusion with fluorescent proteins (A) and various principles of tag–probe labeling (B–E). (B) Protein–ligand interaction (e.g., SNAP-tag® and HaloTag®). A protein tag fused to a target protein is labeled with a ligand conjugated to a fluorophore. (C) Peptide–peptide interaction (e.g., coiled-coil labeling and VIP tags). Two peptides that form a tight heterodimer are used as a combination of a tag fused to the target protein and a probe conjugated with a fluorophore. (D) Metal chelation (e.g., TC-FLAsh™ and Oligo-Asp tag/Zn(II) complex probe). A tag and a probe are cooperative chelators for a metal ion. The tag–metal–probe motif is often multimerized to obtain sufficient binding affinity. (E) Enzymatic reaction (e.g., ACP-tag). A specific site of a tag sequence (substrate 1) is covalently modified with a probe (substrate 2) by an enzymatic reaction. The label size, degree of nonspecific labeling, and simplicity of the labeling procedure differ among the labeling principles.

Figure 2 Coiled-coil labeling. (A) Helical wheel representation of the E3/ K3 coiled-coil heterodimer. White and black arrows indicate hydrophobic interactions (heptad positions *a* and *d*) and electrostatic interactions (heptad positions *e* and *g*) respectively. (B) Labeling of receptors by

the coiled-coil method. E3-tagged  $\beta_2$ -adrenergic receptors ( $\beta_2$ ARs) are quickly labeled after addition of K3/K4 probes conjugated with a fluorescent moiety. In contrast to the image of fluorescent proteins fused at the C-terminal of the receptor (green), coiled-coil labeling is cell-surface specific (red). Reprinted with permission from reference [22]. Copyright (2008) American Chemical Society. (C) Helical wheel representation for the SYNZIP5 (2–44)/SYNZIP6 (16–54) coiled-coil heterodimer [28,30]. The alignment was elucidated from the crystal structure. White arrows indicate hydrophobic interactions (heptad positions *a* and *d*). In addition to electrostatic attractions between the *e* and *g* positions, *a*–*g* interactions between K24–D37 and K31–D44 were observed (black arrow with broken line).

Figure 3 Labeling strategies based on a coiled-coil assembly. (A) Noncovalent labeling. High affinity of the coiled-coil (e.g.  $K_d \sim 6$  nM for E3/K4 pair) enables imaging of target proteins with a high signal/background ratio even without washout of free probes. (B) Crosslinking between tag and probe after coiled-coil assembly. Thiol group of cysteine or amino group of lysine introduced into the tag can be used as the reaction site. (C) Acyl transfer of a fluorescent moiety from the probe to the tag following coiled-coil assembly. Washout of the probe after acyl transfer results in covalent labeling of a small size.

Figure 4 Image analysis of receptor internalization. (A) After incubation with the agonists,  $\beta_2$ -adrenergic receptors ( $\beta_2$ ARs) doubly labeled with pH-dependent fluorophore (green) and pH-independent fluorophore (red) exhibit a significant decrease in the fluorescence intensity ratio green/red due to acidification in endosomes. The degrees of endocytosis (quantified by image analysis) and acidification can be used to characterize the agonists from the fluorescence imaging. (B)(C) Small scale screening of agonists (B) and antagonists (C). In the antagonist assay, the isoproterenol (agonist)-induced acidification and endocytosis were competitively inhibited with the screened compounds. The agonists that promoted receptor internalization, the agonists that did not promote receptor internalization, and the antagonists are represented by solid squares, solid triangles, and solid circles, respectively. Other compounds are represented by open squares. The compounds that exhibited false-positive signals are marked with asterisks. Reprinted with permission from reference [40]. Copyright (2012) American Chemical Society.

Figure 5 FRET detection of the oligomerization of membrane proteins by coiled-coil labeling and confocal imaging with a spectral detector. Fluorescence emission spectra of the cell membrane region under excitation of the FRET donor obtained from cells expressing dimer



standard proteins (metabotropic glutamate receptor) (A) and monomer standard proteins (glycopholin A G83I mutant) (B) at a donor/acceptor labeling ratio of (1/1). Black, green, red, and blue lines indicate the observed spectra, the deconvoluted spectral components of the donor, that of the acceptor, and the spectra expected for directly excited acceptors, respectively. The dimer standard shows sensitized emission from the acceptor (white arrow) due to self-association of the proteins. Reconstructed from reference [53].

Figure 6 Stoichiometric analysis of oligomeric state of membrane proteins. The apparent FRET efficiency based on sensitized acceptor emission was calculated with the equation,  $(\varepsilon_A/\varepsilon_D) \times (F_{AD}-F_A)/F_A$ , where  $\varepsilon_A$  and  $\varepsilon_D$  represent the molar extinction coefficient of the acceptor and donor at donor excitation wavelength, and  $F_{AD}$  and  $F_A$  indicate the acceptor emission in the presence and absence of the donor, respectively. The value is plotted as a function of the donor mole fraction. Solid lines indicate theoretical curves for the monomer ( $N = 1$ ), dimer ( $N = 2$ ), trimer ( $N = 3$ ), tetramer ( $N = 4$ ), and pentamer ( $N = 5$ ). Symbols represent measured values for E3-tagged membrane proteins (M2, M2 protein of influenza A virus; mGluR1b, metabotropic glutamate receptor subtype 1b; GpA\*, glycopholin A G83I mutant;  $\beta_2$ AR,  $\beta_2$ -adrenergic receptor). Reconstructed from reference [53].



Figure 1

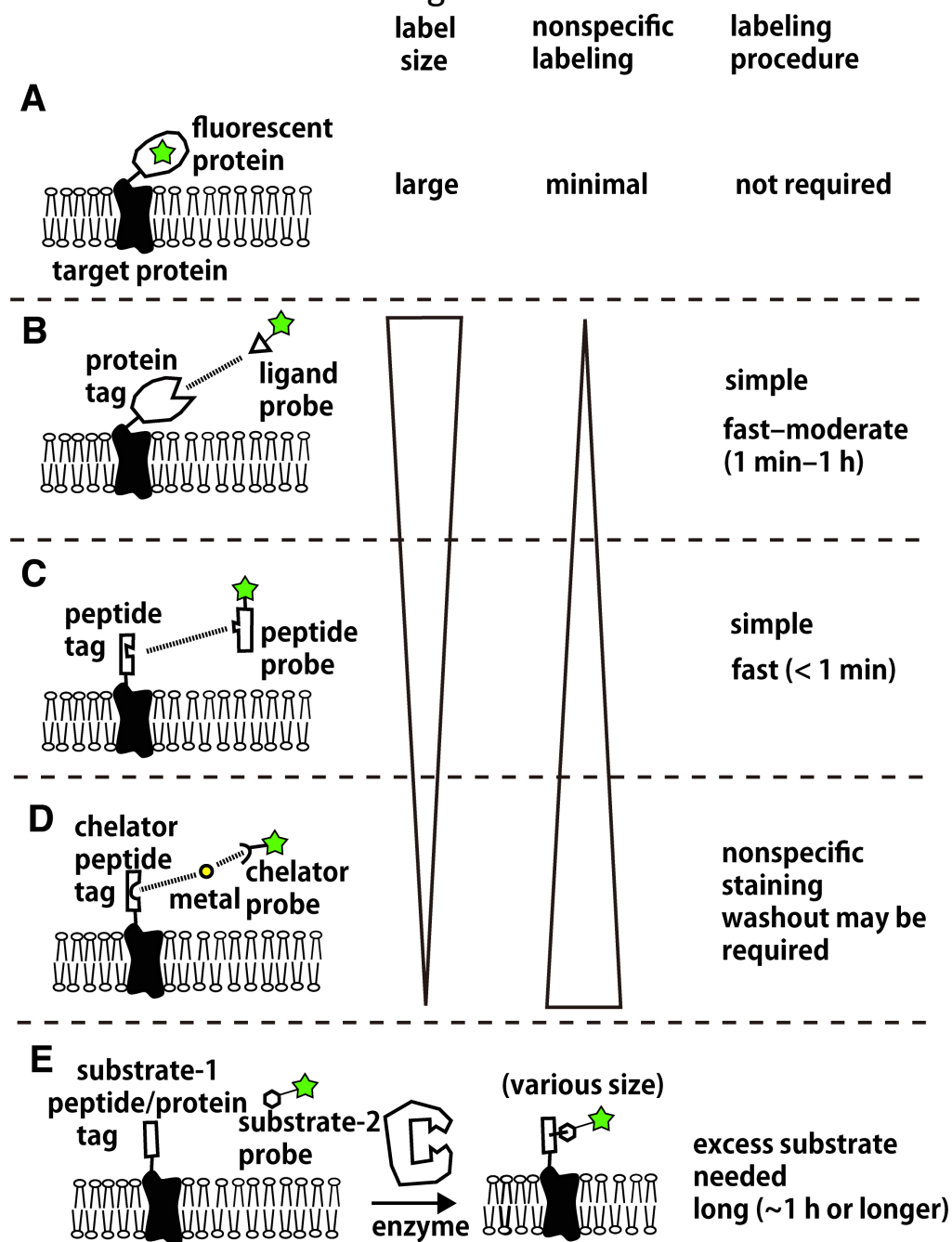


Figure 2

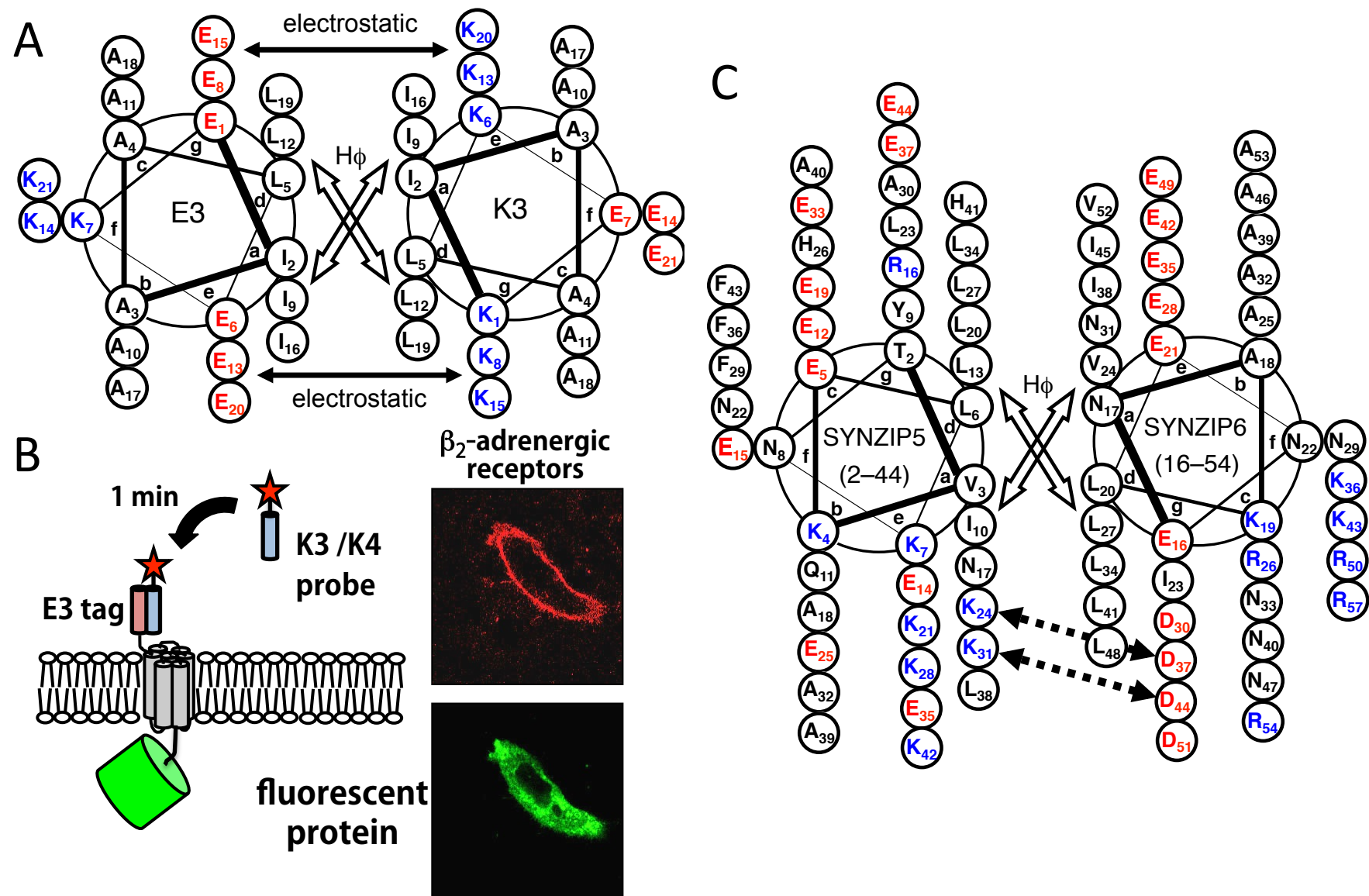
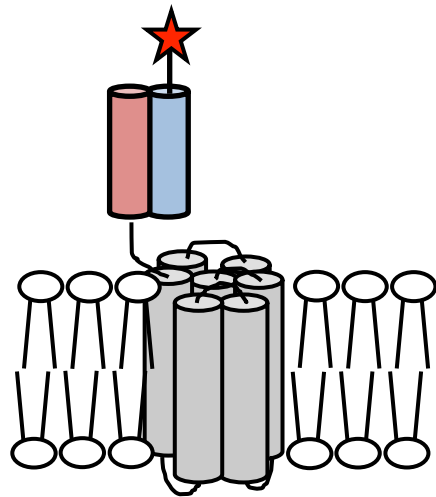
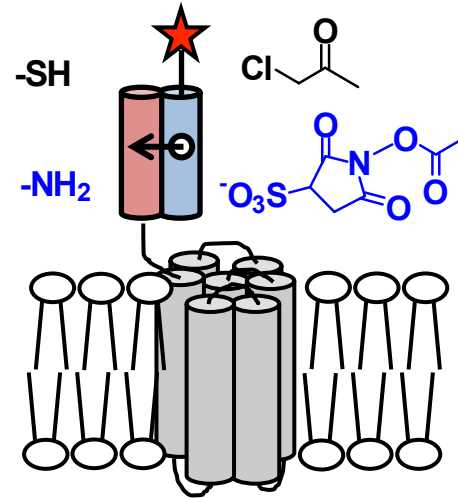


Figure 3

A



B



C

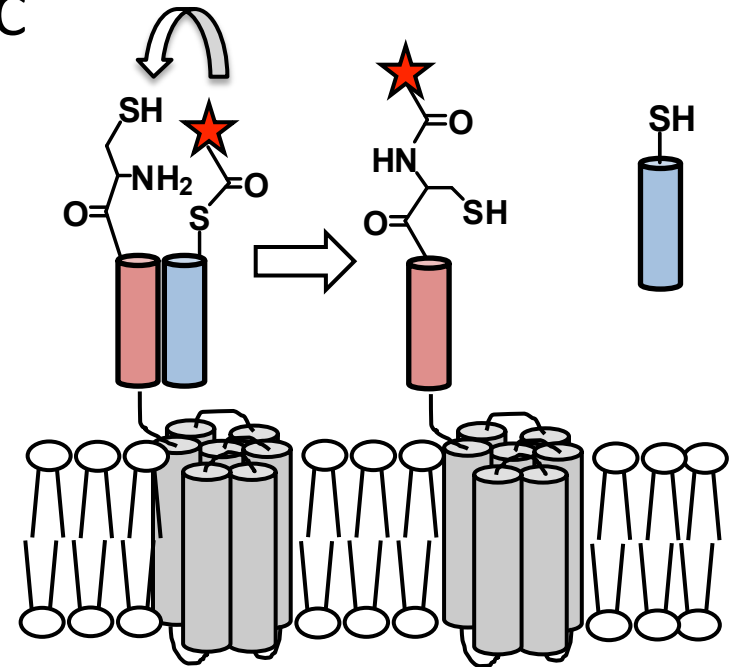


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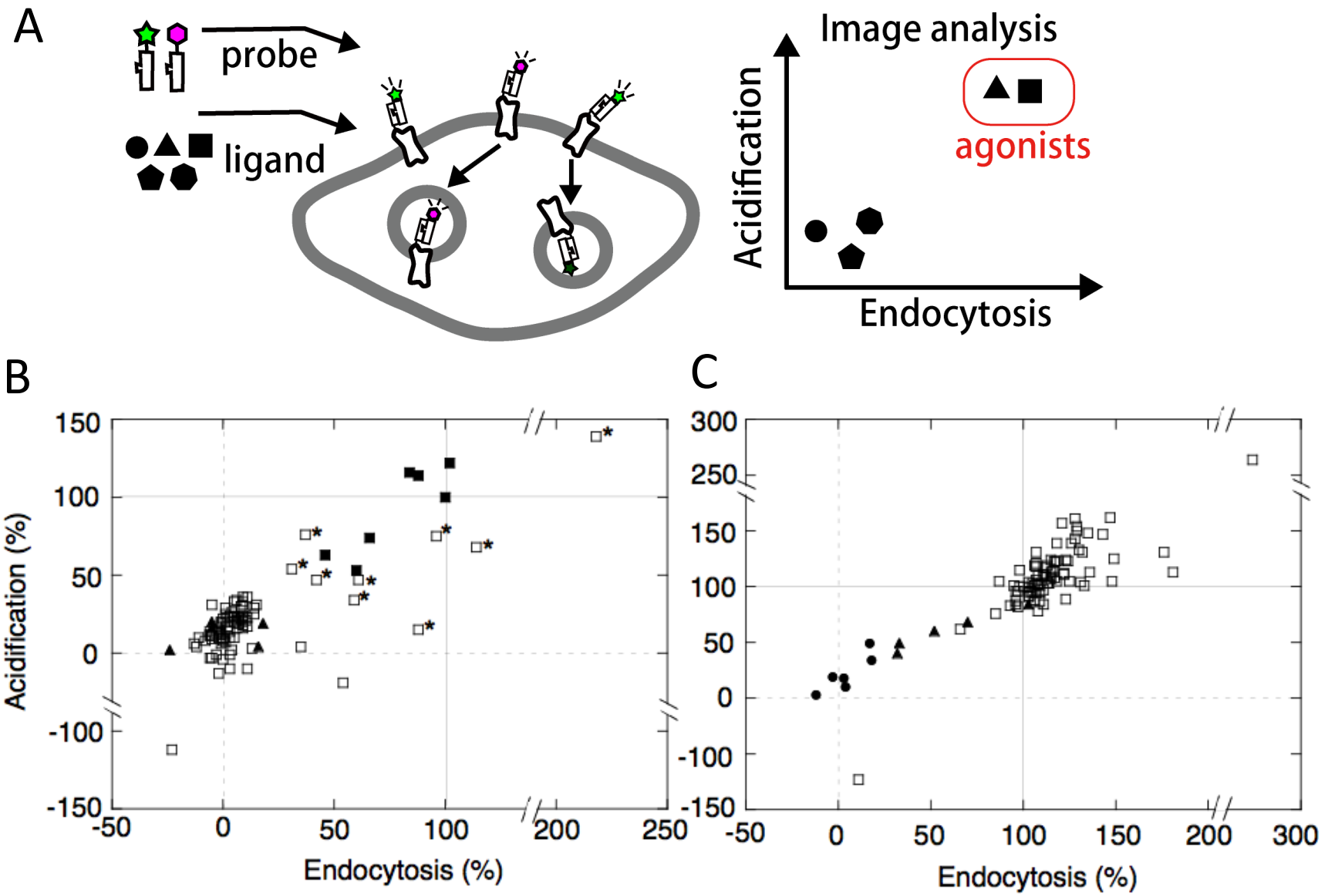


Figure 5

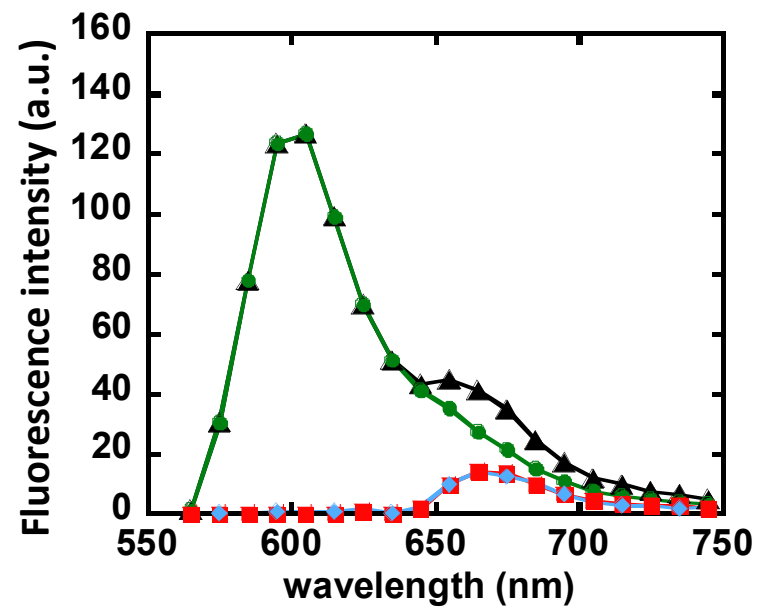
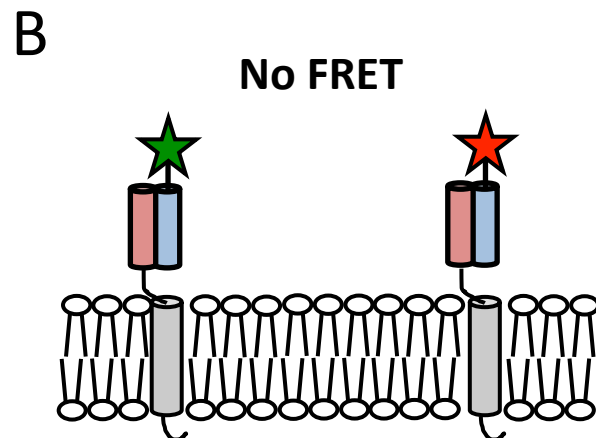
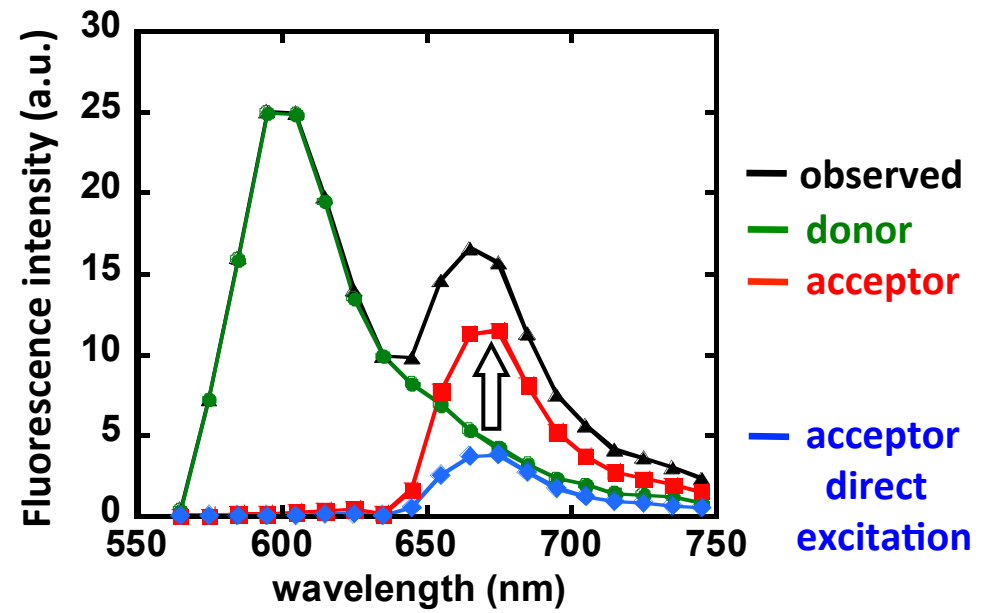
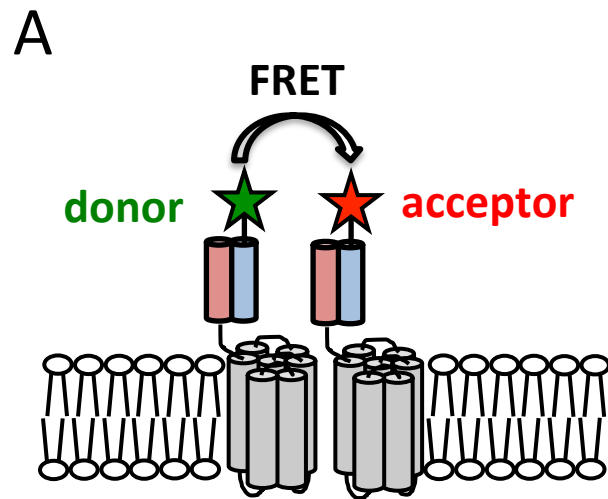


Figure 6

